

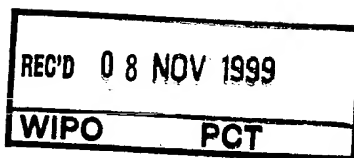


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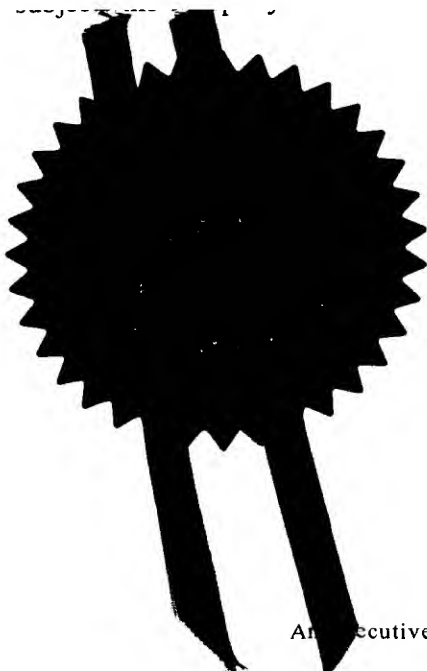
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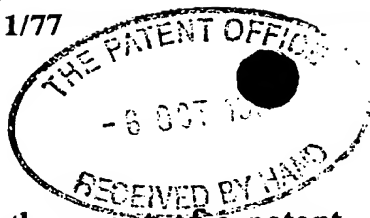
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REP05851GB

2. Patent application number

(The Patent Office will fill in this part)

06 OCT 1998

9821783.9

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Bioprocessing Limited  
Unit 31  
No 1 Industrial Estate  
Medomsley Road  
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Co. Durham, DH8 6SZ  
United Kingdom

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

GB

6122378001

4. Title of the invention

ADSORBENT MEDIUM

5. Name of your agent (if you have one)

GILL JENNINGS & EVERY

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Broadgate House  
7 Eldon Street  
London  
EC2M 7LH

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Country

Priority application number  
(if you know it)

Date of filing  
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Number of earlier application

Date of filing  
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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

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- a) any applicant named in part 3 is not an inventor
  - b) there is an inventor who is not named as an applicant, or
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Description 9

Claim(s) 1

Abstract

Drawing(s)

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Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

Request for preliminary examination and search (*Patents Form 9/77*)

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11. For the Applicant  
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature

Date

6 October 1998

12. Name and daytime telephone number of person to contact in the United Kingdom

PERRY, Robert Edward  
0171 377 1377

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## ADSORBENT MEDIUM

Field of the Invention

This invention relates to an adsorbent medium and to its use in retaining large molecular species, e.g. in the purification of DNA.

Background of the Invention

Cellulosic sponge materials have been used in various forms as the basis of an adsorbent medium, typically after the introduction of ion-exchange groups. For example, GB-A-1226448 discloses the introduction of cross-linking residues into regenerated cellulose, together with or followed by the introduction of ion-exchange groups. The resultant medium can be used, packed in a column. Various physical forms are disclosed.

WO-A-9117830 discloses an adsorbent medium prepared by cross-linking a flexible hydrophilic sponge that contains fibrous, hydrophilic reinforcement, and introducing functional groups. The cross-linking is controlled so that the resulting sponge has a water retention value of 2 to 6. It is proposed that this medium is suitable for the isolation of separation of macro molecules such as proteins, while retaining mechanical strength.

The known adsorbent medium, in addition to its low water retention value, is not suitable because of its inferior flow properties.

A particular area in which an efficient, commercial process is required, for purifying samples, is in the preparation of pharmaceutical grade plasmid DNA for gene therapy. Although many processes have been developed for the large-scale production of recombinant plasmid DNA, e.g. using *E. coli* as the host cell, purification of the product is necessary before the functional gene can be used, i.e. for expression in somatic tissues with the intention of selectively correcting or modulating disease conditions.

Purification of plasmid DNA has traditionally been on a very small scale for research purposes. Purification of plasmid DNA for preclinical toxicology, human clinical

trials, and ultimately for an approved pharmaceutical indication, requires a process that reproducibly meets all the quality and regulatory standards and can be used to purify large quantities of the material economically.

5       The standard method for plasmid DNA purification by molecular biologists has been by caesium chloride/ethidium bromide ultracentrifugation. This method is unacceptable for the production of clinical materials, because it uses mutagenic reagents and is unscalable.

10       Alternative methods for the purification of plasmid DNA have been developed using a combination of chromatography techniques. Problems associated with such techniques have been low capacity of the adsorbents for plasmid DNA, denaturation and breakdown of the DNA  
15       molecule, and losses due to filtration of the feedstock containing plasmid DNA. Problems associated with filtration have been resolved by using the adsorbents in fluidised bed mode, but this requires specially designed hardware for large-scale applications.

20       Summary of the Invention

      This invention is based on the discovery that an adsorbent medium comprising particles of a cellulose sponge material carrying functional groups, can be effectively used to retain species having molecular weight of at least  
25       one million Daltons, e.g. viruses and DNA. In particular, it has been found that an effective adsorbent can be prepared from readily-available sponge material, after removal of fibrous reinforcement.

      The new porous matrix has high capacity for plasmid  
30       DNA, and can be used in a packed bed with unfiltered feedstock. It can be used in any type of chromatographic technique, including ion-exchange, and also affinity and hydrophobic interaction.

Description of the Invention

35       The cellulosic sponge material from which the novel medium is derived, is typically a naturally-occurring polymer such as cellulose or agarose.

Cellulosic sponge materials are commercially available, including hydrophilic, fibrous reinforcement. For the purposes of the present invention, this reinforcement can be removed, or at least substantially removed. The water retention of the medium is generally higher than that described in WO-A-9117830, and is typically greater than 6 ml/g, e.g. up to 10 or 11 ml/g.

The material used in the invention is preferably cross-linked. This may be achieved by known procedures. Similarly, the introduction of functional groups may be achieved in convention manner. Suitable ion-exchange groups are known. It is preferred that the functional groups should bind DNA. They may be derived from tertiary amines such as diethylaminoethane (DEAE), or quaternary amines.

The adsorbed medium of this invention may be made into particles of suitable size by any appropriate technique, e.g. chopping, .... The size of the particles may be heterogeneous; it is not necessary to control its uniformity. The particles will usually be at least 0.1 mm in size, e.g. up to 10 mm or more; a preferred particle size is 0.5 or 1 to 10 mm.

In an illustrative embodiment of the invention, an open structure fibrous polymer adsorbent (HVFM) has been used for the purification of plasmid DNA. In order to demonstrate its suitability, the material was converted into a weak anion-exchanger (DEAE-HVFM) and used to purify plasmid DNA from unfiltered feedstock.

Particulate HVFM was derivatised, up to a level of 1933  $\mu\text{moles per g dry weight}$ , with diethylaminoethane (DEAE). The characteristics of particulate DEAE-HVFM for the purification of plasmid DNA from crude feedstock were determined. The dynamic capacity of DEAE-HVFM for plasmid DNA was  $>1500 \mu\text{g.ml}^{-1}$  and  $1350 \mu\text{g.ml}^{-1}$  at  $88 \text{ cm.h}^{-1}$  (30 column volumes per hour) and  $175 \text{ cm.h}^{-1}$  (60 column volumes per hour), respectively.

Analysis of the purified plasmid DNA was carried out by various methods: it contained no detectable amount of RNase and was not damaged by this purification process. The pressure/flow properties of particulate DEAE-HVFM showed that a flow rate of 116 column volumes per hour can be achieved at a pressure of 2.2 bar, across a column having a bed height of 4 cm and 2.5 cm in diameter.

The water retention value of the particulate DEAE-HVFM is approximately 10 ml/g. A water retention value of 2 to 6 is quoted for HVFM with a uniformly distributed fibrous reinforcement (WO-A-9117830).

The following Examples illustrate the invention.



## Matrix Preparation and Activation

### *Preparation of Particles:*

White HVFM Cloth (18 x 20cm) obtained from Spontex Limited, St Nicholas Quay, Swansea, was washed in tap water at 50°C, followed by purified water, and rolled dry at room temperature. The HVFM was cut into pieces (approximately 2cm square) and the 'nylon' scrim reinforcement was removed. The HVFM pieces were covered with water in a plastic beaker and homogenised using a hand-held Kenwood Blender for approximately 2 minutes. Homogenised HVFM was poured into a standard column and squeezed dry using the plunger of the column.

### *Primary cross linking:*

430mls water and 3.5mls of 1,3, dichloropropanol were mixed and added to the HVFM particles in a 500ml glass beaker and stirred gently to mix. The beaker was covered with non-PVC cling film (four sheets) and 1 layer of aluminium foil and incubated at 60°C in an oven. The reagents were poured off and the matrix washed and squeezed dry using vacuum suction and then used for a column in chromatography column.

### *Derivitization with DEAE*

55.4g of DEAE were dissolved in 122mls purified water and poured onto the HVFM particles in a 500ml glass beaker and gently mixed. 250mls of 5M NaOH were poured evenly onto the HVFM and mixed gently. The beaker was covered in 4 sheets of non-PVC cling film and one sheet of aluminium foil and incubated for 1h. at 60°C in an oven. The reagents were poured off and the particles were dried under vacuum suction. Fresh solutions of 45% (w/v) DEAE and 5M NaOH were added to the HVFM particles as above and incubated again at 60°C. The reagents were poured off and the particles were washed extensively under vacuum suction, followed by squeezing dry in a chromatography column.

## **Analysis of the Ion-exchange HVFM**

### ***Small Ion Capacity:***

A 1ml Omni-fit column (ID 0.66cm) was prepared by hydrating approximately 1g wet weight of DEAE-HVFM with 20mM Tris pH 7.5 and packing it into the column using the column plunger. The column was then equilibrated with 0.2M NaCl (adjusted to pH 11.0 with NaOH) at  $0.5\text{mlmin}^{-1}$ . 35 ml of 12mM HCl was then washed through the column at  $0.5\text{mlmin}^{-1}$ , 1ml fractions were collected and the pH of each fraction measured. The column was then unpacked and the DEAE-HVFM dried to constant weight at  $50^{\circ}\text{C}$  in an oven. A graph of pH against  $\mu\text{moles}$  of HCl applied to the column was plotted and the point at which the pH dropped sharply to approximately pH 3.0 was used to calculate the level to which the sponge had been derivitized with DEAE.

The small ion capacity for the HVFM particles was calculated from the titration curve to be  $1933\mu\text{moles DEAE/g HVFM}$  (dry weight).

### ***Pressure Flow Characteristics:***

A 2.5cm I.D Omni-fit column was used to pack columns of DEAE-HVFM. The backpressure across the column was monitored with increasing flow rate until the pressure became excessive.

## **Purification of Plasmid DNA**

### ***Feedstock:***

The feedstock containing recombinant plasmid DNA was a gift from Cobra Therapeutics. Crude plasmid DNA was harvested from the bioreactor and bag-filtered. The material was frozen and shipped to Bioprocessing. The material was defrosted in small aliquots and any precipitate was removed using a filter paper.

### ***Static Capacities for plasmid DNA:***

HVFM particles were packed into 1ml glass columns and equilibrated with 20mM Tris pH 7.5. 20-30mls of feedstock were loaded onto the columns. The columns were washed with 5mls of 20mM Tris pH 7.5 and eluted with 5mls each of 20mM Tris pH 7.5 containing 0.5M, 1.0M and 2.0M NaCl. Samples were prepared for analysis by Plasmix capture (see below).

### *Dynamic Capacity for plasmid DNA:*

1 ml Omni-fit columns (ID 0.66cm) were packed with the matrix and equilibrated with 20mM tris pH 7.5 at 0.5mlmin<sup>-1</sup> (88cmh<sup>-1</sup>). 50mls of feedstock were filtered through Whatman N°1 filter paper and loaded onto the column at 0.5mlmin<sup>-1</sup>. The column was then washed (at 0.5mlmin<sup>-1</sup>) with 10mls 20mM tris pH 7.5 and eluted with 10mls of 0.5M NaCl/ 20mM tris pH 7.5, 20mls of 1.0M NaCl/ 20mM tris pH 7.5, and 10mls of 2.0M NaCl/ 20mM tris pH 7.5 at 0.5mlmin<sup>-1</sup>, 1.0ml fractions were collected throughout. In a further experiment the equilibration, loading, washing and elution of the DEAE-HVFM were all carried out at 1.0mlmin<sup>-1</sup>.

### *Analysis of Breakthrough:*

#### 1. Plasmix Capture

140µl of fractions were taken, 350µl of Plasmix purification resin was added and mixed in Plasmix filters. The filters were placed on the plasmix vacuum manifold and vacuum applied (resin with bound DNA is retained on the filter). The resin was washed twice with 400 µl Plasmix washing solution and the vacuum continued for an additional 5 minutes to dry the filter. The plasmix filters were then transferred to eppendorf centrifuge tubes and the DNA resuspended by adding 50µl of 40mM tris/ 1mM EDTA pH 7.6 at 70°C and standing at room temperature for 60 minutes. Plasmid DNA was eluted by centrifugation for 20minutes at 13K rpm in an eppendorf centrifuge.

#### 2. Agarose Gel-electrophoresis.

0.6% (w/v) agarose gels in 40mM tris /1mM EDTA pH 7.5 were cast. Samples were prepared by mixing 30µl of the plasmid DNA with 3µl loading buffer and applying 30µl of each sample to the wells. Gels were run in 2L 40mM tris/ 1mM EDTA pH 7.6 at 250mA for approximately 2.5h. Gels were stained with 400ml ethidium bromide in 2L H<sub>2</sub>O for approximately 45minutes, illuminated on a transilluminator and photographed (Kodak SBX film and White print film).

### *Analysis of Residuals*

#### 1. Gel filtration on Sephacryl S-500 HR.

A column of Sephacryl S-500 HR (43 x 0.66cm) was packed and equilibrated with 50mM tris/ 5mM EDTA / 0.5M NaCl pH 7.6 at 0.25mlmin<sup>-1</sup>. Samples (100µl) were loaded and eluted with 50mM tris/ 5mM EDTA / 0.5M NaCl pH 7.6 at 0.25mlmin<sup>-1</sup> for 90 minutes. OD 260 / 280nm was monitored.

#### 2. Protein. – Silver Stained SDS-polyacrylamide gel electrophoresis (PAGE)

Feedstock samples and fractions from IEX plasmid DNA dynamic capacity experiments were used undiluted in the ratio 0.57 sample: 0.33 NuPAGE

sample buffer : 0.1 reducing agent. Ribonuclease A (type XII A,  $M_r$  13,500) was used as a standard with between 100 and 1000ng loaded. Samples and standards were heated at 70°C for 10 minutes before loading 25µl aliquots onto 4-14% Bis Tris NuPAGE gels (Novex). Gels were run in either MES or MOPS buffer systems at 200V constant for approximately 50 minutes. Bands were visualised by silver staining.

3. RNA and Low  $M_r$  DNA – Agarose gel electrophoresis with SYBR green II staining.

1 % (w/v) agarose gels in 40mM tris/ 1mM EDTA pH 7.6. Feedstock and IEX plasmid DNA dynamic capacity fractions were used without prior Plasmix capture. Samples (30µl) were mixed with 3µl loading solution and 30µl applied to the wells of the gel. Gels were run at 250mA in 2L 40mM tris/ 1mM EDTA pH 7.6 for approximately 30 minutes. Gels were stained in 1L 89mM tris/ 89mM Boric acid/ 1mM EDTA pH 8.0 containing 200µl stock SYBR green II DMSO solution (i.e. 1:5000 dilution of SYBR green II) for 40 minutes.

*Integrity of plasmid DNA*

Plasmid DNA was precipitated from feedstock and IM NaCl elutions from DEAE-HVFM as follows:-

0.7ml propan 2 ol was added to 1 ml solution containing plasmid DNA and the mixture was incubated for 30mins at -20°C. The solution was centrifuged for 30mins, 13Krpm, room temp. in a microfuge. The resulting precipitate was retained and the supernatant discarded. 0.5ml of 70% (v/v) ethanol was added and the solution was centrifuged for 30 mins, 13K, room temp. in a microfuge. The resulting pellet was retained and the supernatant discarded. The pellet was allowed to air dry for 30 minutes at room temperature. 400µl of 40mM tris/ 1mM EDTA pH 7.6 was added to resuspend the pellet. This solution was serially diluted with 40mM tris/ 1mM EDTA pH 7.6 for electrophoresis. 0.6% (w/v) agarose gels were cast as above. 3µl loading buffer was added to 30µl aliquots of resuspended plasmid DNA, 30µl was loaded onto the gel. Gels were run and stained with ethidium bromide as above.

**Results**

*Dynamic Capacity*

No breakthrough of DNA was seen on agarose gel electrophoresis with ethidium bromide staining after loading 50mls of feedstock (30µg plasmid DNA per ml) at 0.5mlmin<sup>-1</sup> (88cmh<sup>-1</sup>) indicating a dynamic capacity for plasmid DNA of > 1.5mgml<sup>-1</sup> (Plate 1a). Increasing the flow rate to 1mlmin<sup>-1</sup> resulted in ethidium bromide staining of DNA after 45mls of 30µgml<sup>-1</sup> i.e. a dynamic capacity of 1350µgml<sup>-1</sup>.

### *Analysis of Residuals*

#### 1. Gel filtration on Sephacryl S-500 HR.

CsCl purified plasmid DNA was excluded from Sephacryl S-500. Feedstock containing plasmid eluted as a small peak (approximately 1% of the OD 260 absorbing material) at the void volume of the column (23 minutes, 5.75mls) and a large peak (99%) close to the total volume of the column (60 minutes, 15mls) (Figure 4). After IEX fractionation on DEAE-HVFM the 20mM tris pH 7.5 wash contained only low  $M_r$  material eluting after 64 minutes (16mls), this material was not RNA or low  $M_r$  DNA – see below under SYBR green II staining. The 0.5M NaCl elution contained low  $M_r$  material (59mins, 14.75mls) and no plasmid DNA. The 1.0M NaCl elution contained plasmid DNA (5.4% of material) at the void volume of the column and low  $M_r$  material eluting after 55mins (13.75mls).

#### 2. Residual RNA

Residual RNA was visualised by 1% (w/v) agarose gel electrophoresis with SYBR green II staining. In fractions from a plasmid DNA breakthrough experiment with DEAE-HVFM RNA was visible in the feedstock, not in the breakthrough or the 20mM tris pH 7.5 wash, and present in the 0.5M and 1.0M NaCl elutions. The RNA in the 0.5M NaCl elution migrated marginally further than that in the 1.0M NaCl elution suggesting smaller species of RNA, this is consistent with the gel filtration results.

#### 3. Residual Protein.

The presence of residual protein was investigated with silver stained SDS-PAGE on 4-12% Bis/Tris gels. Ribonuclease A (RNase  $M_r$  13,500) was the most heavily stained protein in the feedstock and therefore probably a good indicator of the presence of residual protein. The heavy staining at the origin of

down to 100ng. Strong RNase staining was seen in the breakthrough fractions from DEAE-HVFM and this protein was also detected in the 20mM tris wash. No RNase was present in the 0.5M or 1.0M NaCl elutions suggesting that very little residual protein is present, also there was heavy staining at the origin of the gel which was probably due to the DNA eluted.

### *Integrity of Plasmid DNA.*

DNA was precipitated with propanol from feedstock and the 1.0M NaCl elutions from DEAE-HVFM and subjected to 0.6% (w/v) agarose gel electrophoresis with ethidium bromide staining (Plate 4). CsCl purified plasmid was included as another control. The ratio of covalently closed circular DNA to open circular was visually the same for the feedstock and the 1.0M NaCl elutions from DEAE-HVFM, evidence that no damage had been done to the DNA by binding and elution from the matrix.

CLAIMS

1. An adsorbent medium comprising particles of a cellulosic sponge material carrying functional groups.
2. A medium according to claim 1, wherein the sponge material is free of fibrous reinforcement.
3. A medium according to claim 1 or claim 2, which has a water retention value of greater than 6 ml/g.
4. A medium according to any preceding claim, wherein the sponge material is a naturally-occurring polymer.
5. A medium according to claim 4, wherein the natural polymer is cellulose or agarose.
6. A medium according to any preceding claim, wherein the functional groups bind DNA.
7. A medium according to claim 6, wherein the functional groups are derived from DEAE.
8. A medium according to any preceding claim, wherein the particles are 0.5 to 10 mm in size.
9. A medium according to any preceding claim, capable of retaining species having a molecular weight of at least one million Daltons.
10. A medium according to any preceding claim, wherein the sponge material is cross-linked.
11. A method for purifying DNA in an aqueous sample, which comprises passing the sample through a medium according to any preceding claim.

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